



## **Biochemical evidence that regulation of Ero1 activity in human cells does not involve the isoform-specific cysteine 262**

Hansen, Henning Gram; Søltoft, Cecilie Lützen; Schmidt, Jonas Damgård; Birk, Julia; Appenzeller-Herzog, Christian; Ellgaard, Lars

*Published in:*  
Bioscience Reports

*DOI:*  
[10.1042/BSR20130124](https://doi.org/10.1042/BSR20130124)

*Publication date:*  
2014

*Document version*  
Publisher's PDF, also known as Version of record

*Citation for published version (APA):*  
Hansen, H. G., Søltoft, C. L., Schmidt, J. D., Birk, J., Appenzeller-Herzog, C., & Ellgaard, L. (2014). Biochemical evidence that regulation of Ero1 activity in human cells does not involve the isoform-specific cysteine 262. *Bioscience Reports*, 34(2). <https://doi.org/10.1042/BSR20130124>



## OPEN ACCESS

# Biochemical evidence that regulation of Ero1 $\beta$ activity in human cells does not involve the isoform-specific cysteine 262

Henning G. HANSEN\*<sup>1</sup>, Cecilie L. SØLTOFT\*, Jonas D. SCHMIDT\*<sup>2</sup>, Julia BIRK†, Christian APPENZELLER-HERZOG\*† and Lars ELLGAARD\*<sup>3</sup>

\*Department of Biology, University of Copenhagen, 2200 Copenhagen, Denmark

†Department of Pharmaceutical Sciences, University of Basel, 4056 Basel, Switzerland

## Synopsis

In the ER (endoplasmic reticulum) of human cells, disulfide bonds are predominantly generated by the two isoforms of Ero1 (ER oxidoreductin-1): Ero1 $\alpha$  and Ero1 $\beta$ . The activity of Ero1 $\alpha$  is tightly regulated through the formation of intramolecular disulfide bonds to help ensure balanced ER redox conditions. Ero1 $\beta$  is less tightly regulated, but the molecular details underlying control of activity are not as well characterized as for Ero1 $\alpha$ . Ero1 $\beta$  contains an additional cysteine residue (Cys<sup>262</sup>), which has been suggested to engage in an isoform-specific regulatory disulfide bond with Cys<sup>100</sup>. However, we show that the two regulatory disulfide bonds in Ero1 $\alpha$  are likely conserved in Ero1 $\beta$  (Cys<sup>90</sup>–Cys<sup>130</sup> and Cys<sup>95</sup>–Cys<sup>100</sup>). Molecular modelling of the Ero1 $\beta$  structure predicted that the side chain of Cys<sup>262</sup> is completely buried. Indeed, we found this cysteine to be reduced and partially protected from alkylation in the ER of living cells. Furthermore, mutation of Cys<sup>100</sup> – but not of Cys<sup>262</sup> – rendered Ero1 $\beta$  hyperactive in cells, as did mutation of Cys<sup>130</sup>. Ero1 $\beta$  hyperactivity induced the UPR (unfolded protein response) and resulted in oxidative perturbation of the ER redox state. We propose that features other than a distinct pattern of regulatory disulfide bonds determine the loose redox regulation of Ero1 $\beta$  relative to Ero1 $\alpha$ .

**Key words:** disulfide-bond formation, endoplasmic reticulum oxidoreductin-1 (Ero1), redox regulation, unfolded protein response (UPR)

Cite this article as: Hansen, H.G., Søltoft, C.L., Schmidt, J.D., Birk, J., Appenzeller-Herzog, C. and Ellgaard, L. (2014) Biochemical evidence that regulation of Ero1 $\beta$  activity in human cells does not involve the isoform-specific cysteine 262. *Biosci. Rep.* **34**(2), art:e00103.doi:10.1042/BSR20130124

## INTRODUCTION

In the ER (endoplasmic reticulum), optimal redox conditions are maintained to facilitate formation of native disulfide bonds in secretory proteins. In mammalian cells, disulfide bonds are mainly generated by Ero1 (ER oxidoreductin-1) [1,2]. Proteins of the Ero1 family comprise two conserved di-cysteine active sites [3] (Figure 1). The so-called inner active site sits adjacent to a FAD moiety inside a four-helix bundle, whereas the outer active site (containing the two ‘shuttle’ cysteines) is located on a flexible loop region [4,5]. The inner active site is oxidized by molecular oxygen via FAD, which leads to generation of

hydrogen peroxide [6–8]. In turn, the inner active site oxidizes the shuttle cysteines by thiol–disulfide exchange [9]. The shuttle cysteines then oxidize active-site cysteines in members of the PDI (protein disulfide-isomerase) family [2, 8, 10–12]. As the final step in the Ero1–PDI disulfide relay, PDIs introduce disulfide bonds into newly synthesized proteins in the ER [13].

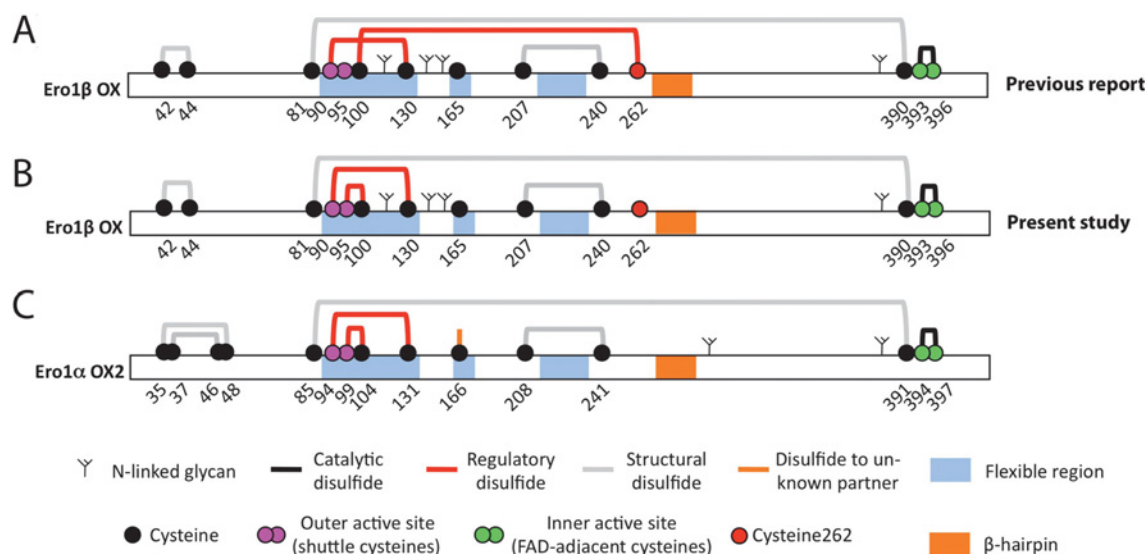
Two isoforms of Ero1 have been identified in nearly all vertebrates studied so far [14] including humans: Ero1 $\alpha$  and Ero1 $\beta$  [15,16]. Whereas Ero1 $\alpha$  is widely expressed, Ero1 $\beta$  is predominantly found in select tissues, such as the pancreas and salivary gland [16,17]. Both Ero1 isoforms are up-regulated by the UPR (unfolded protein response), which is a transcriptional and translational programme that is induced by accumulation of

**Abbreviations:** AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; ATF6 $\alpha$ , activating transcription factor 6 $\alpha$ ; BiP, immunoglobulin heavy-chain-binding protein; DTT, dithiothreitol; Dox, doxycycline; ER, endoplasmic reticulum; Ero1, endoplasmic reticulum oxidoreductin-1; Ero1 $\alpha$ -WT, wild-type Ero1 $\alpha$ ; HEK-293 cells, human embryonic kidney cells; HERP, homocysteine-induced endoplasmic reticulum (ER) protein; NEM, N-ethylmaleimide; PDI, protein disulfide-isomerase; PERK, PKR (double-stranded-RNA-dependent protein kinase)-like endoplasmic reticulum kinase; TCA, trichloroacetic acid; UPR, unfolded protein response.

<sup>1</sup> Present address: Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2970 Hørsholm, Denmark.

<sup>2</sup> Present address: Department of International Health, Immunology and Microbiology, University of Copenhagen, 2200 Copenhagen, Denmark.

<sup>3</sup> To whom correspondence should be addressed (email: lellgaard@bio.ku.dk).



**Figure 1** Disulfide bonds in Ero1 $\beta$  and Ero1 $\alpha$

(A) Schematic representation of the proposed disulfide pattern in the OX redox form of Ero1 $\beta$  as reported by Wang et al. [7]. (B) Proposed disulfide bond pattern in Ero1 $\beta$  based on the present study. (C) Disulfide bond pattern in Ero1 $\alpha$  OX2 verified by mass spectrometry [23,24] and crystallography [4]. The cysteine residues are shown in black, magenta (outer active site), green (inner active site) and red (Cys<sup>262</sup> in Ero1 $\beta$ ) with amino acid numbering. Disulfide bonds are depicted as thick grey (likely structural), black (active site) or red (reported regulatory function; Ero1 $\beta$  [7,32] and this study, Ero1 $\alpha$  [4,23,24,47]) lines. The thick orange line at Cys<sup>166</sup> indicates the connection to a potential (but unidentified) disulfide partner. The flexible regions are coloured in light blue and fork-like branches depict predicted high-mannose N-linked glycans. The  $\beta$ -hairpin region shown to interact with PDI is coloured orange.

misfolded proteins in the ER (designated ER stress). The UPR seeks to restore ER homeostasis, for example by decreasing the ER protein load through translational arrest and in parallel up-regulating chaperones to assist folding [18]. PERK [PKR (double-stranded-RNA-dependent protein kinase)-like endoplasmic reticulum kinase], Inositol-requiring enzyme 1 $\alpha$  (Ire1 $\alpha$ ) and ATF6 $\alpha$  (activating transcription factor 6 $\alpha$ ) are the three transmembrane transducers that initiate ER-to-nucleus signalling upon ER stress. Whereas the PERK pathway is involved in up-regulating Ero1 $\alpha$  [19], Ero1 $\beta$  is induced by the XBP1s transcription factor (which is activated by Ire1 $\alpha$ ) [20] and ATF6 $\alpha$  [21].

Apart from the first three cysteines in Ero1 $\alpha$  (Cys<sup>35</sup>, Cys<sup>37</sup> and Cys<sup>46</sup>), the 12 additional cysteines are conserved in the vertebrate branch of the Ero1 family [22,23]. In addition to these 12 cysteines, human Ero1 $\beta$  contains a cysteine residue in position 262 (Cys<sup>262</sup>) (Figure 1). The disulfide pattern in Ero1 $\alpha$  has been mapped by mass spectrometry [23,24] and crystallography [4] (Figure 1). In contrast to Ero1 from *Saccharomyces cerevisiae* (Ero1p) [5], the Ero1 $\alpha$  shuttle cysteines (Cys<sup>94</sup> and Cys<sup>99</sup>) can engage in regulatory disulfide bonds with non-active-site cysteines (Cys<sup>131</sup> and Cys<sup>104</sup>, respectively) [23,25]. The presence of these two disulfide bonds blocks the outer active site and thus inhibits the activity of Ero1 $\alpha$  [23,25]. In the cell, formation and reduction of these inhibitory disulfide bonds depend on the redox state of PDI [23]. This gives rise to a tightly regulated homeostatic feedback mechanism where Ero1 $\alpha$  is only active when oxidized PDI is scarce [23]. Since the ER glutathione redox buffer influences the redox state of PDI [26,27], Ero1 $\alpha$  activity is modulated by the

ratio between oxidized and reduced glutathione mediated through PDI [28]. Similarly, redox regulation of Ero1p in *S. cerevisiae* is also influenced by the redox state of PDI and glutathione [29–31]. Moreover, the ratio between oxidized and reduced glutathione is tightly balanced in the ER in human cells, which is at least in part a consequence of the Ero1 feedback regulation [2].

In comparison with Ero1 $\alpha$ , Ero1 $\beta$  activity does not seem to be as tightly regulated. Whereas overexpressed Ero1 $\alpha$ -WT (wild-type Ero1 $\alpha$ ) is predominantly inactive and therefore has a subtle effect on the redox state of the PDI homologue ERp57 [23,24], overexpression of Ero1 $\beta$ -WT hyperoxidizes ERp57, i.e. leads to a larger fraction of the molecules with active-site cysteines in the disulfide-bonded state [23,32]. Similarly, Ero1 $\beta$ -WT is more active than Ero1 $\alpha$ -WT in an *in vitro* oxidation assay performed with PDI as the substrate [7]. On non-reducing SDS-PAGE gels exogenous Ero1 $\beta$  expressed in mammalian cells migrates as two distinct redox species, with the distribution between the faster migrating (OX) and slower migrating species (Red) varying between experiments [23,33,34]. Similar to Ero1 $\alpha$  [25], an initial shift from the OX to the Red species of Ero1 $\beta$  was observed during the catalysis of thioredoxin oxidation *in vitro* [7]. When thioredoxin was completely oxidized, the redox state of Ero1 $\beta$  reverted to the OX species [7]. Thus, Ero1 $\beta$  activity is also regulated by intramolecular disulfides.

In Ero1 $\alpha$ , a cysteine-to-alanine mutant of Cys<sup>104</sup> and Cys<sup>131</sup> (Ero1 $\alpha$ -C104A/C131A) displays hyperactivity since it can no longer form the two regulatory disulfides, but retains the two residues of the outer active site, Cys<sup>94</sup> and Cys<sup>99</sup> [24,25].

Recently, we showed that overexpression in human cells of the equivalent Ero1 $\beta$  mutant (Ero1 $\beta$ -C100A/C130A) gave rise to more pronounced hyperoxidation of ERp57 relative to overexpression of Ero1 $\beta$ -WT [32], suggesting that the regulatory mechanism is shared for Ero1 $\alpha$  and Ero1 $\beta$ . However, Ero1 $\beta$  contains an additional cysteine residue (Cys<sup>262</sup>), which is not present in Ero1 $\alpha$ . A disulfide bond between Cys<sup>100</sup> and Cys<sup>262</sup> was recently proposed to be present in Ero1 $\beta$  purified from *Escherichia coli* [7]. Moreover, Ero1 $\beta$ -C100A displayed slowed oxidation kinetics relative to Ero1 $\beta$ -WT [7], suggesting that the presence of the proposed Cys<sup>100</sup>–Cys<sup>262</sup> disulfide bond increases Ero1 $\beta$  activity. On this background, we decided to further investigate the interplay between intramolecular disulfide bonds and regulation of activity in Ero1 $\beta$ .

## MATERIALS AND METHODS

### Primers and plasmids

Human Ero1 $\beta$ -myc6his ([16]; a gift from R. Sitia, Milan) cloned into the pcDNA5/FRT/TO vector [23] was used as a template for QuikChange mutagenesis (Stratagene) to introduce Cys-to-Ala mutations. The following primer was used to generate the C262A mutation (only the sense strand is shown): C262A 5'-GACTTCATGCTAGCATCAATTTACATCTAGCCGCAAATTATCTTTTGG-3'. The C100A and C130A mutations have been described before [32]. All plasmids were sequenced to confirm the correct DNA sequence of the inserts.

### Cell culture

Dox (doxycycline)-inducible Flp-In T-REx HEK-293 (Life Technologies) cell lines were generated and grown as previously described [23]. Ero1 $\beta$  expression was induced for 24 h (unless otherwise stated) using 1  $\mu$ g/ml Dox (Sigma). For ER stress induction, cells were treated with either 5  $\mu$ M thapsigargin (Sigma) or 2.5  $\mu$ g/ml tunicamycin (Sigma) for the indicated time.

### Sample preparation and AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid) modification

Cells were treated with NEM (*N*-ethylmaleimide) and subsequently lysed as described elsewhere [35]. The AMS (Life Technologies) modification protocol has been described previously [35]. Reduced and oxidized control lysates were obtained from cells treated with 10 mM DTT (dithiothreitol) or 5 mM diamide (both Sigma) for 5 min at 37°C in full growth medium.

### Antibodies and Western blotting

The following mouse monoclonal antibodies were used:  $\alpha$ His (Tetra-His, Qiagen),  $\alpha$ myc (9E10, Covance),  $\alpha\beta$ -actin (AC-15, Sigma). The rabbit polyclonal antisera used were:  $\alpha$ BiP (G8918,

Sigma),  $\alpha$ ERp57 (a gift from A. Helenius, Zürich, Switzerland),  $\alpha$ HERP (a gift from L. Hendershot, Memphis, TN, U.S.A.). Western blotting was performed as previously described [24]. The shown Western blots are representative of at least two independent experiments.

### Redox state analysis of Ero1 $\beta$ by TCA (trichloroacetic acid) precipitation and alkylation of free thiols

Cells cultivated to 60–80% confluency in 6 cm dishes were washed twice in PBS. They were then concomitantly lysed and precipitated by incubation in 10% (v/v) TCA for 15 min on ice. Cells were transferred to an Eppendorf tube, centrifuged (16 100 g, 4°C, 15 min) and the supernatant was discarded. Pellets were washed once in ice-cold acetone, centrifuged (16 100 g, 4°C, 15 min) and resuspended in 100  $\mu$ l 100 mM Tris–HCl pH 7.0, 8% (v/v) glycerol, 2% (w/v) SDS, 10% dimethyl sulfoxide, 0.01% (w/v) bromocresol purple and 20 mM NEM. Samples were neutralized by drop-wise addition of 1 M Tris–HCl, pH 7.5, 2% SDS until samples turned purple (bromocresol purple changes colour between pH 5.2 and 6.8). The pellets were subsequently dissolved by sonication, incubated at RT in the dark for 1 h and the redox state of Ero1 $\beta$  was determined by non-reducing Western blotting.

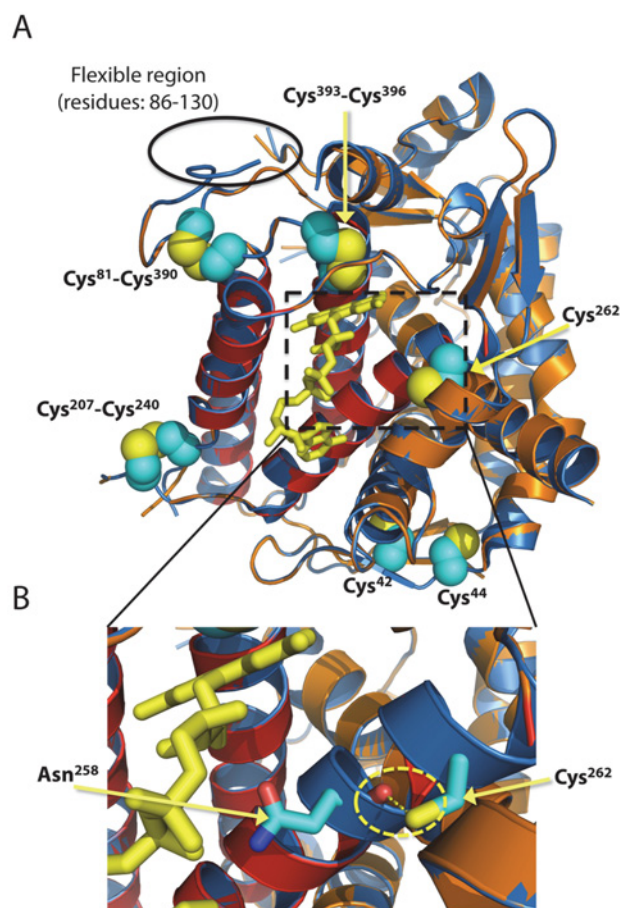
## RESULTS

### Structure homology modelling of Ero1 $\beta$ predicts Cys<sup>262</sup> to be buried in the structure

The amino acid sequences of Ero1 $\beta$  are highly conserved between orthologues (Supplementary Figure S1 available at <http://www.biosciencerep.org/bsr/034/bsr034e103add.htm>). Thus, potential roles of cysteine residues in regulatory disulfide bonds based on evolutionary conservation could not be inferred from a multiple sequence alignment. Instead, we used structure homology modelling of Ero1 $\beta$  to assess the proposed disulfide patterns in the protein (Figures 1A and 1B). The protein structure prediction software SWISS-MODEL [36] was used to predict the three-dimensional structure of Ero1 $\beta$  based on the crystal structure of inactive Ero1 $\alpha$ , a mutant in essence corresponding to the OX2 form ([4]; PDB ID: 3AHR) (Figure 2A). The sequences of mature Ero1 $\alpha$  and Ero1 $\beta$  are highly similar [14] with a sequence identity of 65%. As expected from the high sequence conservation, the  $\alpha$ -helical fold in Ero1 $\alpha$  was predicted to be preserved in Ero1 $\beta$  including the four-helix bundle involved in FAD binding (Figure 2A, red-coloured  $\alpha$ -helices). The structure of the flexible region (residues 86–130) comprising the proposed Cys<sup>90</sup>–Cys<sup>130</sup> or the Cys<sup>95</sup>–Cys<sup>100</sup> disulfide bonds could not be reliably modelled (Figure 2A).

In contrast to the cysteines in the flexible region, Cys<sup>262</sup> is located at the end of a conserved helix [14], which is part of the four-helix bundle (Figure 2A). Moreover, Cys<sup>262</sup> is positioned close





**Figure 2 Structural model of Ero1 $\beta$**

(A) Superimposition of the structure of inactive Ero1 $\alpha$  (PDB ID: 3ahr; [4]) and a structural model of Ero1 $\beta$ . The latter was homology modelled based on the structure of inactive Ero1 $\alpha$  using the SWISS-MODEL software [36]. Predicted flexible regions (see Figure 1) are not depicted. The approximate position of the flexible region comprising residues 86–130 in Ero1 $\beta$  is indicated. In Ero1 $\beta$ , the  $\alpha$ -helices of the four-helix bundle are shown in red, the remainder of the molecule in orange and the cysteine residue side chains are depicted as spheres (yellow: sulfur, cyan: carbon) with amino acid numbering indicated. Ero1 $\alpha$  is depicted in blue and the FAD moiety (stick model) in yellow. (B) Zoom of the structural overlay in (A). The predicted hydrogen bond (encircled) between the Ero1 $\beta$  Cys<sup>262</sup> SH-group and the carbonyl O (red) of Asn<sup>258</sup> is shown by the broken yellow line. In the model, the distance between the S and O atoms is 2.74 Å. This figure was created in PyMOL (<http://www.pymol.org>).

to a protruding  $\beta$ -hairpin, which is critical for the interaction with PDI [37]. The equivalent residue in Ero1 $\alpha$  (Ser<sup>263</sup>) is completely buried. Similarly, in the Ero1 $\beta$  model, Cys<sup>262</sup> is predicted to have a relative accessible surface area of 0 (as calculated by the ASAView software [38] and the GETAREA method [39]), which strongly suggests that Cys<sup>262</sup> in Ero1 $\beta$  is buried in the native structure. Moreover, the side chain –SH (Cys<sup>262</sup>)/–OH (Ser<sup>263</sup>) is predicted to form a hydrogen bond with the backbone carbonyl group of Asn<sup>258</sup>/Asn<sup>259</sup>, respectively (Figure 2B). This hydrogen bond seems to be part of a conserved hydrogen bond network, including hydrogen bonds from the side chain of Asn<sup>258</sup>/Asn<sup>259</sup>

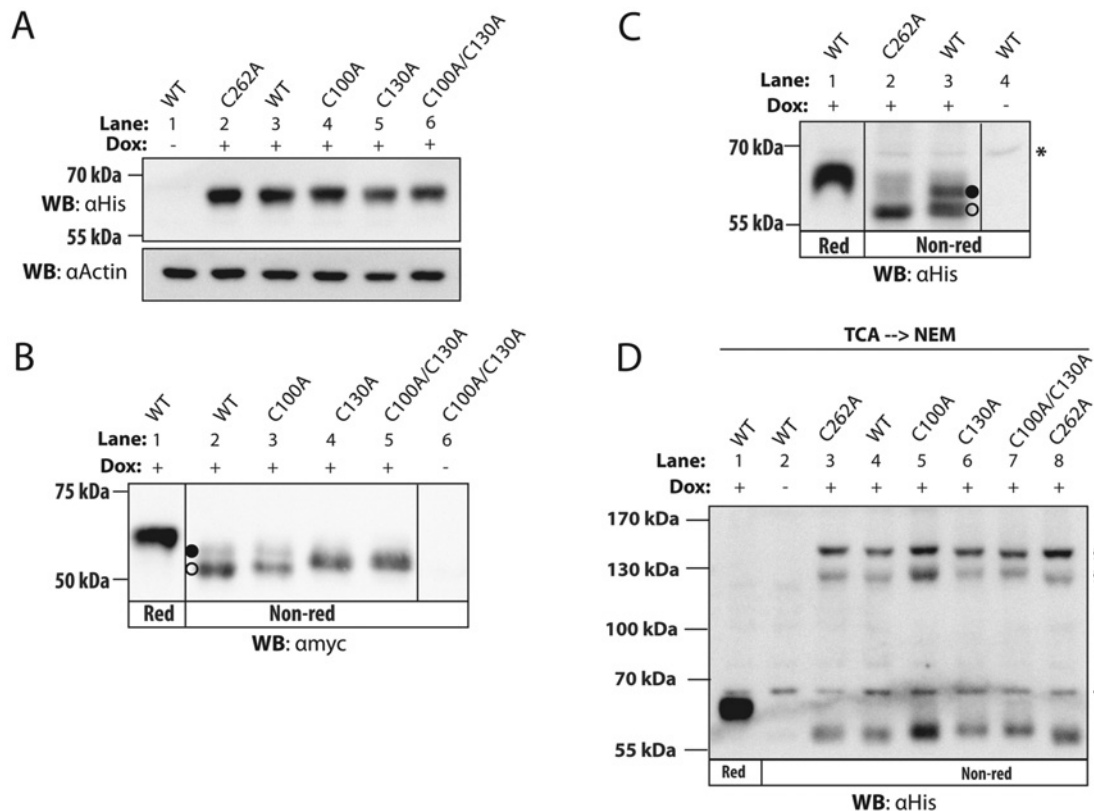
to FAD, which helps stabilize the structure in the vicinity of the bound cofactor. Finally, we also note that in *Xenopus tropicalis* Ero1 $\beta$ , a serine residue is found in place of Cys<sup>262</sup> (Supplementary Figure S1), indicating that a cysteine is not strictly necessary at this position as may have been expected if it played an important function in regulating the activity of the enzyme.

### SDS–PAGE mobility of Ero1 $\beta$ mutants suggests conservation of regulatory disulfide bonds in Ero1 $\alpha$ and Ero1 $\beta$

To investigate the structural importance of intramolecular disulfide bonds in human Ero1 $\beta$ , we expressed Ero1 $\beta$  cysteine mutants in human cells and analysed the mobility of these mutants by non-reducing SDS–PAGE. Apart from already established stable cell lines for ectopic inducible expression of Ero1 $\beta$ -WT [23] and Ero1 $\beta$ -C100A/C130A [32], we generated three new inducible cell lines for the following mutants: Ero1 $\beta$ -C100A, Ero1 $\beta$ -C130A and Ero1 $\beta$ -C262A. As compared with Ero1 $\beta$ -WT, Ero1 $\beta$ -C100A and Ero1 $\beta$ -C262A showed similar expression levels, whereas the expression levels of Ero1 $\beta$ -C130A and Ero1 $\beta$ -C100/130A were lower (Figure 3A). Importantly, none of the cell lines overexpressing Ero1 $\beta$  mutants of Cys<sup>100</sup> and/or Cys<sup>130</sup>, which turned out to be hyperactive (see below), expressed more protein than the Ero1 $\beta$ -WT-expressing cell line.

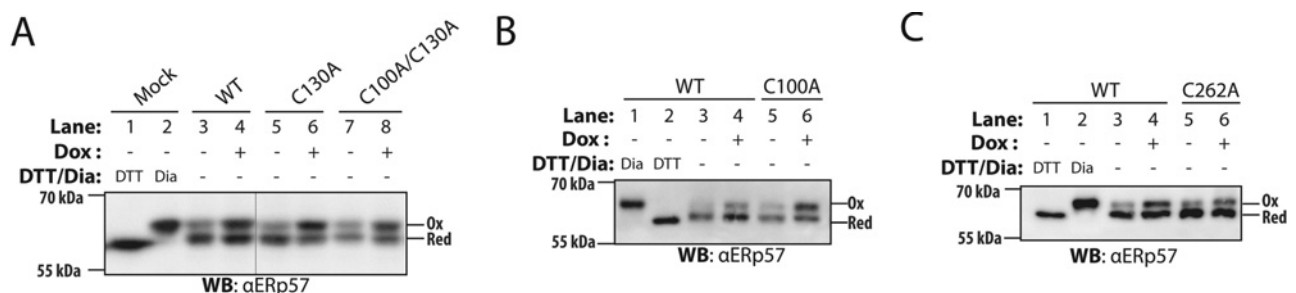
As previously observed [33], the monomeric form of exogenous Ero1 $\beta$ -WT migrated as two distinct redox species (Red and OX) when cells were treated with NEM to alkylate free thiols *in situ* prior to lysis (Figure 3B, lane 2 and Figure 3C, lane 3). However, upon TCA precipitation with subsequent NEM treatment, monomeric Ero1 $\beta$ -WT migrated as one redox species (Figure 3D, lane 4). TCA precipitation rapidly quenches thiol–disulfide exchange reactions and denatures proteins, enabling alkylation of thiols buried in the native structure [40]. When cells are *in situ* NEM-treated, approximately 20% of the cellular protein thiols have been shown to be inaccessible to NEM [41]. Such NEM inaccessibility is thought to be a consequence of these thiols being buried in the native structure [40]. We therefore suggest that inefficient alkylation of (a) free thiol(s) buried in the structure of Ero1 $\beta$  gives rise to rearrangement of disulfide bonds upon denaturation, leading to the appearance of the Red Ero1 $\beta$  redox form (Figures 3B and 3C). Conversely, when all free thiols are efficiently alkylated, Ero1 $\beta$ -WT is preserved as a single redox species visible on SDS–PAGE gels (Figure 3D).

The SDS–PAGE mobility of the Ero1 $\beta$  variants on non-reducing gels (Figure 3D) is consistent with Ero1 $\beta$  having a similar pattern of disulfide bonds as Ero1 $\alpha$  (Figures 1B and 1C). We were able to detect a relatively small migration shift between Ero1 $\beta$ -WT and Ero1 $\beta$ -C100A (Figure 3D, lanes 4–5) suggesting that Cys<sup>100</sup> is not engaged in a long-range disulfide bond. In contrast, a larger shift was observed upon mutation of Cys<sup>130</sup> (Figure 3D, lanes 6–7) consistent with removal of the longer-ranging Cys<sup>90</sup>–Cys<sup>130</sup> disulfide bond. No redox species of Ero1 $\beta$ -C100A co-migrated with Ero1 $\beta$ -C130A (Figure 3D, lanes 5–6), suggesting that the Cys<sup>90</sup>–Cys<sup>130</sup> disulfide bond is intact in Ero1 $\beta$ -C100A.



**Figure 3** SDS-PAGE mobility of Ero1 $\beta$  variants suggests that Ero1 $\alpha$  and Ero1 $\beta$  share their sets of regulatory disulfide bonds

(A) Expression of His- and Myc-tagged Ero1 $\beta$  variants was induced with Dox for 24 h and cells were NEM treated to alkylate free thiols. Equal amounts of protein from lysates were analysed by reducing SDS-PAGE and Western blotting using  $\alpha$ His (Ero1 $\beta$ ) and  $\alpha$ Actin (loading control) to compare expression levels of Ero1 $\beta$  variants. (B,C) Cell lysates were obtained as in (A). The SDS-PAGE mobility of the Ero1 $\beta$  variants was analysed under non-reducing (Non-red) or reducing (Red) conditions by  $\alpha$ myc or  $\alpha$ His Western blotting. The open and filled circles indicate the previously described OX and Red redox forms of Ero1 $\beta$  WT [33], respectively, and vertical hairlines denote removal of lanes. Asterisk denotes a background band. (D) Expression of Ero1 $\beta$  variants was induced as in (A). Cells were subjected to TCA precipitation to rapidly quench thiol-disulfide exchange reactions and to denature cellular proteins. Precipitates were redissolved in a buffer containing NEM to alkylate free thiols. Subsequently, the SDS-PAGE mobility of the Ero1 $\beta$  variants was analysed under non-reducing conditions by  $\alpha$ His Western blotting. Section signs (§) indicate possible Ero1 $\beta$  mixed-disulfide dimeric species and the asterisk (\*) denotes a background band.



**Figure 4** Hyperoxidation of ERp57 is intensified by removal of regulatory disulfide bonds in Ero1 $\beta$

(A–C) Where indicated, expression of Ero1 $\beta$  variants was induced with Dox for 24 h. Prior to lysis, cells were treated with NEM to alkylate free thiols. After cell lysis, cysteines present in disulfides were reduced and decorated with AMS. Such AMS modification of active-site cysteines originally present in the oxidized state gives rise to slower SDS-PAGE mobility compared with the (NEM-decorated) pool of ERp57 containing reduced active-site cysteines. The cellular redox state of ERp57 was visualized by Western blotting. DTT and Diamide (Dia) treated-cells were used to show the mobility of fully oxidized (Ox) and reduced (Red) ERp57. A vertical hairline denotes removal of lanes.

A fraction of Ero1 $\beta$  is present as a disulfide-bonded homodimer in human cells [33] and when expressed in bacteria [7]. Moreover, Ero1 $\beta$  engages in heterodimeric mixed-disulfide species with PDI and ERp44 in human cells [42]. The possible dimeric species involving Ero1 $\beta$ -WT and Ero1 $\beta$ -C262A were similar (Figure 3D, lanes 3–4), suggesting that Cys<sup>262</sup> is not involved in formation of mixed-disulfide dimeric species. Notably, Ero1 $\beta$ -C262A did not migrate slower than Ero1 $\beta$ -WT (Figure 3D, lanes 3–4), suggesting that Cys<sup>262</sup> is not engaged in a long-range disulfide bond. Instead, Ero1 $\beta$ -C262A was present exclusively as the OX redox species in lysates from cells treated *in situ* with NEM (Figure 3C, lane 2), and as a single redox species co-migrating with Ero1 $\beta$ -WT in lysates from cells subjected to TCA precipitation (Figure 3D, lane 3). This clearly suggests that non-native *ex vivo* disulfide shuffling in lysates of *in situ* NEM-treated cells observed for Ero1 $\beta$ -WT (Figures 3B and 3C) depends on the presence of Cys<sup>262</sup>, and that this residue is inaccessible to NEM in the native structure.

Based on these results, we propose that the regulatory disulfide bonds in Ero1 $\alpha$  (Cys<sup>94</sup>–Cys<sup>131</sup> and Cys<sup>99</sup>–Cys<sup>104</sup>) are conserved in Ero1 $\beta$  (Cys<sup>90</sup>–Cys<sup>130</sup> and Cys<sup>95</sup>–Cys<sup>100</sup>) and that Cys<sup>262</sup> constitutes a poorly accessible free thiol in the native structure. The deduced disulfide pattern in the OX redox form of Ero1 $\beta$  is shown in Figure 1(B).

### Removal of either of the regulatory disulfide bonds increases the activity of Ero1 $\beta$ in cells

We next wanted to assess the relative contribution of the proposed disulfide bonds (Cys<sup>90</sup>–Cys<sup>130</sup> and Cys<sup>95</sup>–Cys<sup>100</sup>) to the regulation of Ero1 $\beta$  activity. First, we analysed the cellular redox state of ERp57, as assessed by differential alkylation of the active-site cysteines. This assay probes the ratio of ERp57 molecules with active-site cysteines in the oxidized and reduced state, respectively, and has been used routinely in the field as readout for changes in the ER redox environment [23,27,43]. Mutation of Cys<sup>100</sup> and Cys<sup>130</sup> alone or in combination increased the hyperoxidizing effect of Ero1 $\beta$  on the redox state of ERp57 relative to Ero1 $\beta$ -WT (Figures 4A and 4B). This suggests that both disulfide bonds (Cys<sup>90</sup>–Cys<sup>130</sup> and Cys<sup>95</sup>–Cys<sup>100</sup>) are involved in inhibiting the activity of Ero1 $\beta$ . Consistent with Cys<sup>262</sup> not being involved in regulation of Ero1 $\beta$  activity, overexpression of Ero1 $\beta$ -C262A showed only a minor hyperoxidizing effect on ERp57 (Figure 4C). It should be noted that consistent with our previous studies, and as noted before [2], the ERp57 redox state differed between individual experiments likely reflecting physiological variations. This, however, does not affect the overall conclusions concerning the relative oxidizing effects of overexpressing different Ero1 $\beta$  variants.

We recently showed that a deregulated Ero1 $\alpha$  mutant (Ero1 $\alpha$ -C104A/C131A) markedly activated the UPR as a result of its increased oxidase activity when overexpressed in HEK-293 cells (human embryonic kidney cells) [24]. To study whether overexpression of Ero1 $\beta$  also induces the UPR, we analysed the protein levels of the two established UPR targets, BiP (immunoglobulin heavy-chain-binding protein) and HERP (homocysteine-induced

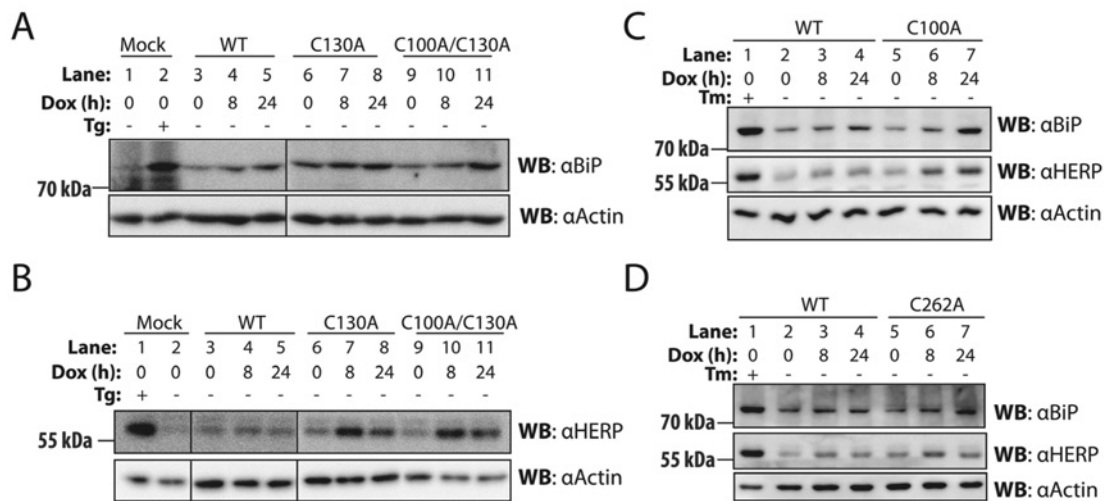
ER protein) [44]. As expected from the loose regulation of Ero1 $\beta$  (Figure 4), BiP and HERP levels were moderately increased upon expression of Ero1 $\beta$ -WT (Figures 5A–5D). These effects were more pronounced upon mutation of Cys<sup>100</sup> and Cys<sup>130</sup> alone or in combination (Figures 5A–5C), correlating with the impact of these mutants on the redox state of ERp57 (Figures 4A and 4B). Overexpression of Ero1 $\beta$ -C262A showed a similar degree of UPR induction as Ero1 $\beta$ -WT (Figure 5D). These findings suggested that Cys<sup>262</sup> is not involved in regulating Ero1 $\beta$  activity, and is in keeping with our proposed disulfide pattern of Ero1 $\beta$  (Figure 1B).

## DISCUSSION

Tight regulation of Ero1 $\alpha$  activity is important to maintain balanced ER redox conditions [23–25]. We propose that the regulatory disulfide bonds in Ero1 $\alpha$  and Ero1 $\beta$  are conserved (Figures 1B and 1C). This conclusion is based on several lines of evidence, including molecular modelling of the Ero1 $\beta$  structure (Figure 2), SDS–PAGE mobility analysis of Ero1 $\beta$  mutants (Figures 3B–3D) and ER redox (Figure 4) and ER stress readouts (Figure 5). Overall, the findings that overexpression of Ero1 $\beta$  mutants devoid of Cys<sup>100</sup> and/or Cys<sup>130</sup> induces the UPR, hyperoxidizes ERp57 and that Ero1 $\beta$ -C100A/C130A hyperoxidizes an ER-localized glutathione sensor [32], indicate that the underlying mechanism is likely to involve an oxidizing perturbation of the ER redox environment, which in turn results in protein misfolding and therefore activation of the UPR.

In a previous study [7], Ero1 $\beta$ -C262A purified from *E. coli* displayed a prominent slow-migrating redox species when compared with Ero1 $\beta$ -WT by non-reducing SDS–PAGE, indicating the loss of a long-range disulfide. Furthermore, analysis of tryptic fragments supported the presence of a Cys<sup>100</sup>–Cys<sup>262</sup> disulfide bond. Finally, the Ero1 $\beta$ -C100A mutant was less active *in vitro* than Ero1 $\beta$ -WT, suggesting that the presence of the proposed Cys<sup>100</sup>–Cys<sup>262</sup> disulfide bond positively regulates the activity of Ero1 $\beta$ .

Here, we expressed Ero1 $\beta$  (and mutants thereof) in its native environment in the ER of human cells and reached the conclusion that a disulfide bond between Cys<sup>100</sup> and Cys<sup>262</sup> is not likely to form. Thus, mutation of Cys<sup>100</sup> rendered Ero1 $\beta$  hyperactive and overexpression of Ero1 $\beta$ -C262A showed effects comparable to Ero1 $\beta$ -WT overexpression. We also provide two-fold evidence that Cys<sup>262</sup> is a solvent inaccessible residue in the native structure of Ero1 $\beta$ . First, a fraction of Ero1 $\beta$ -WT molecules rearrange into a redox species that migrates as the Red form upon *in situ* NEM treatment in a Cys<sup>262</sup>-dependent manner, suggesting that NEM cannot gain access to Cys<sup>262</sup> under native conditions. Secondly, a homology model of Ero1 $\beta$  based on the crystal structure of Ero1 $\alpha$  places Cys<sup>262</sup> in a non-solvent exposed site in a highly conserved  $\alpha$ -helix. Collectively, these findings strongly support the conclusion that Cys<sup>262</sup> does not engage in an intramolecular disulfide bond with Cys<sup>100</sup>. To verify the proposed



**Figure 5** Absence of regulatory disulfide bonds in Ero1 $\beta$  increases induction of the unfolded protein response (UPR)

(A–D) Expression of Ero1 $\beta$  variants was induced with doxycycline (Dox) for the indicated periods of time. Expression levels of BiP and HERP were analysed by Western blotting using  $\alpha$ Actin as loading control. The mock cell line is stably transfected with an empty vector. Cells were either treated with 5  $\mu$ M thapsigargin (Tg) for 18 h (A) and 6 h (B) or treated with 2.5  $\mu$ g/ml tunicamycin (Tm) for 20 h (C, D) to generate positive control lysates for induction of the UPR.

disulfide pattern, we also sought to map the intramolecular disulfides in Ero1 $\beta$  purified from human cells by mass spectrometry, as has previously been achieved for Ero1 $\alpha$  [23]. Unfortunately, the results obtained by this approach were ambiguous (H. G. Hansen, L. Ellgaard and F. Hubálek, unpublished work), which was likely a result of disulfide bond scrambling in the course of sample preparation.

Using TCA precipitation and subsequent NEM treatment, we demonstrated that the Red form of Ero1 $\beta$  [33] is likely an artefact of inefficient thiol alkylation, indicating that overexpressed Ero1 $\beta$  is present solely as the OX redox species. Unfortunately, the redox state of endogenous Ero1 $\beta$  assessed by SDS–PAGE mobility under non-reducing conditions is currently unknown. Moreover, we currently do not know why Ero1 $\beta$  migrates 5–7 kDa faster than Ero1 $\alpha$  on non-reducing SDS–PAGE gels [23], even though the predicted molecular mass of mature Ero1 $\beta$  is only 1–2 kDa smaller than the corresponding mass of mature Ero1 $\alpha$ . Since deglycosylation of Ero1 $\beta$  gives rise to a more pronounced mobility shift on SDS–PAGE gels as compared with Ero1 $\alpha$  [16], the presence of N-linked glycans cannot explain the unexpectedly large difference in SDS–PAGE mobility between Ero1 $\alpha$  and Ero1 $\beta$ .

As Ero1 $\alpha$  and Ero1 $\beta$  likely share their sets of regulatory disulfide bonds, features other than a distinct pattern of disulfide bonds must determine the loose redox regulation of Ero1 $\beta$  relative to Ero1 $\alpha$ . Mutation of the Cys<sup>394</sup>–Phe–Lys–Cys<sup>397</sup> inner active site sequence of Ero1 $\alpha$  to the Ero1 $\beta$  sequence (Cys<sup>393</sup>–Asp–Lys–Cys<sup>396</sup>) substantially increases the oxidase activity of Ero1 $\alpha$  [7]. This suggests that Asp<sup>394</sup> in Ero1 $\beta$  contributes to the apparently loose redox regulation of Ero1 $\beta$  relative to Ero1 $\alpha$ .

As previously proposed [22], the loose regulation of Ero1 $\beta$  activity relative to Ero1 $\alpha$  could be explained by a higher reduc-

tion potential of the regulatory disulfide bonds in Ero1 $\beta$ . The high expression of Ero1 $\beta$  in the pancreas and salivary gland indicates a specific role of the protein in secretory tissues. Accordingly, oxidative folding of pro-insulin is impeded in pancreatic islet cells derived from Ero1 $\beta$ -compromised mice, an effect that is not exacerbated by concomitantly compromising Ero1 $\alpha$  function [45]. However, increasing disulfide-bond formation by exogenous Ero1 $\alpha$  expression stimulates oxidative folding of pro-insulin [46]. These observations suggest that the loose regulation of Ero1 $\beta$  activity could have evolved to optimally support the high demand of disulfide bonds in secretory tissues.

#### AUTHOR CONTRIBUTION

Henning Hansen and Lars Ellgaard designed and supervised the experimental work. Henning Hansen, Cecilie Søtoft, Jonas Schmidt, Julia Birk and Christian Appenzeller-Herzog performed the experiments. Henning Hansen and Lars Ellgaard wrote the paper, and Christian Appenzeller-Herzog contributed to revision of the paper.

#### ACKNOWLEDGEMENTS

We thank K. L. Doherty for excellent technical assistance, G. Nielsen and F. Hubálek for kind technical help and guidance in our attempts to map the disulfide bonds in Ero1 $\beta$  by mass spectrometry and A. Helenius and L. Hendershot for sharing reagents.

#### FUNDING

This work was supported by the Lundbeck Foundation [grant number 2009-3653 (to L.E.)]. H.G.H. was a recipient of a Ph.D. stipend from the Faculty of Science at the University of Copenhagen, and an EliteForsk Travel Stipend from the Danish Ministry of Science,





Innovation, and Higher Education [grant number 10-093459]. C.A.-H. is an Ambizione grantee of the Swiss National Science Foundation [grant number 142964].

## REFERENCES

- Rutkevich, L. A. and Williams, D. B. (2012) Vitamin K epoxide reductase contributes to protein disulfide formation and redox homeostasis within the endoplasmic reticulum. *Mol. Biol. Cell* **23**, 2017–2027
- Appenzeller-Herzog, C., Riemer, J., Zito, E., Chin, K. T., Ron, D., Spiess, M. and Ellgaard, L. (2010) Disulphide production by Ero1alpha-PDI relay is rapid and effectively regulated. *EMBO J.* **29**, 3318–3329
- Bertoli, G., Simmen, T., Anelli, T., Molteni, S. N., Fesce, R. and Sitia, R. (2004) Two conserved cysteine triads in human Ero1alpha cooperate for efficient disulfide bond formation in the endoplasmic reticulum. *J. Biol. Chem.* **279**, 30047–30052
- Inaba, K., Masui, S., Iida, H., Vavassori, S., Sitia, R. and Suzuki, M. (2010) Crystal structures of human Ero1alpha reveal the mechanisms of regulated and targeted oxidation of PDI. *EMBO J.* **29**, 3330–3343
- Gross, E., Kastner, D. B., Kaiser, C. A. and Fass, D. (2004) Structure of Ero1p, source of disulfide bonds for oxidative protein folding in the cell. *Cell* **117**, 601–610
- Gross, E., Sevier, C. S., Heldman, N., Vitu, E., Bentzur, M., Kaiser, C. A., Thorpe, C. and Fass, D. (2006) Generating disulfides enzymatically: reaction products and electron acceptors of the endoplasmic reticulum thiol oxidase Ero1p. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 299–304
- Wang, L., Zhu, L. and Wang, C. C. (2011) The endoplasmic reticulum sulfhydryl oxidase Ero1beta drives efficient oxidative protein folding with loose regulation. *Biochem. J.* **434**, 113–121
- Wang, L., Li, S. J., Sidhu, A., Zhu, L., Liang, Y., Freedman, R. B. and Wang, C. C. (2009) Reconstitution of human Ero1-Lalpha/protein-disulfide isomerase oxidative folding pathway *in vitro*. Position-dependent differences in role between the a and a' domains of protein-disulfide isomerase. *J. Biol. Chem.* **284**, 199–206
- Sevier, C. S. and Kaiser, C. A. (2006) Disulfide transfer between two conserved cysteine pairs imparts selectivity to protein oxidation by Ero1. *Mol. Biol. Cell.* **17**, 2256–2266
- Tu, B. P. and Weissman, J. S. (2002) The FAD- and O(2)-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum. *Mol. Cell* **10**, 983–994
- Frand, A. R. and Kaiser, C. A. (2000) Two pairs of conserved cysteines are required for the oxidative activity of Ero1p in protein disulfide bond formation in the endoplasmic reticulum. *Mol. Biol. Cell* **11**, 2833–2843
- Mezghrani, A., Fassio, A., Benham, A., Simmen, T., Braakman, I. and Sitia, R. (2001) Manipulation of oxidative protein folding and PDI redox state in mammalian cells. *EMBO J.* **20**, 6288–6296
- Appenzeller-Herzog, C. and Ellgaard, L. (2008) The human PDI family: versatility packed into a single fold. *Biochim. Biophys. Acta* **1783**, 535–548
- Araki, K. and Inaba, K. (2012) Structure, mechanism, and evolution of Ero1 family enzymes. *Antioxid. Redox Signal.* **16**, 790–799
- Cabibbo, A., Pagani, M., Fabbri, M., Rocchi, M., Farmery, M. R., Bulleid, N. J. and Sitia, R. (2000) ERO1-L, a human protein that favors disulfide bond formation in the endoplasmic reticulum. *J. Biol. Chem.* **275**, 4827–4833
- Pagani, M., Fabbri, M., Benedetti, C., Fassio, A., Pilati, S., Bulleid, N. J., Cabibbo, A. and Sitia, R. (2000) Endoplasmic reticulum oxidoreductin 1-beta (ERO1-Lbeta), a human gene induced in the course of the unfolded protein response. *J. Biol. Chem.* **275**, 23685–23692
- Ramming, T. and Appenzeller-Herzog, C. (2012) The physiological functions of mammalian endoplasmic oxidoreductin 1: on disulfides and more. *Antioxid. Redox Signal.* **16**, 1109–1118
- Walter, P. and Ron, D. (2011) The unfolded protein response: from stress pathway to homeostatic regulation. *Science* **334**, 1081–1086
- Marciniak, S. J., Yun, C. Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H. P. and Ron, D. (2004) CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev.* **18**, 3066–3077
- Shoulders, M. D., Ryno, L. M., Genereux, J. C., Moresco, J. J., Tu, P. G., Wu, C., Yates, J. R., 3rd, Su, A. I., Kelly, J. W. and Wiseman, R. L. (2013) Stress-independent activation of XBP1s and/or ATF6 reveals three functionally diverse ER proteostasis environments. *Cell Rep.* **3**, 1279–1292
- Adachi, Y., Yamamoto, K., Okada, T., Yoshida, H., Harada, A. and Mori, K. (2008) ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. *Cell Struct. Funct.* **33**, 75–89
- Tavender, T. J. and Bulleid, N. J. (2010) Molecular mechanisms regulating oxidative activity of the Ero1 family in the endoplasmic reticulum. *Antioxid. Redox Signal.* **13**, 1177–1187
- Appenzeller-Herzog, C., Riemer, J., Christensen, B., Sørensen, E. S. and Ellgaard, L. (2008) A novel disulphide switch mechanism in Ero1alpha balances ER oxidation in human cells. *EMBO J.* **27**, 2977–2987
- Hansen, H. G., Schmidt, J. D., Søltøft, C. L., Ramming, T., Geertz-Hansen, H. M., Christensen, B., Sørensen, E. S., Juncker, A. S., Appenzeller-Herzog, C. and Ellgaard, L. (2012) Hyperactivity of the ero1alpha oxidase elicits endoplasmic reticulum stress but no broad antioxidant response. *J. Biol. Chem.* **287**, 39513–39523
- Baker, K. M., Chakravarthi, S., Langton, K. P., Sheppard, A. M., Lu, H. and Bulleid, N. J. (2008) Low reduction potential of Ero1alpha regulatory disulphides ensures tight control of substrate oxidation. *EMBO J.* **27**, 2988–2997
- Molteni, S. N., Fassio, A., Ciriolo, M. R., Filomeni, G., Pasqualetto, E., Fagioli, C. and Sitia, R. (2004) Glutathione limits Ero1-dependent oxidation in the endoplasmic reticulum. *J. Biol. Chem.* **279**, 32667–32673
- Jessop, C. E. and Bulleid, N. J. (2004) Glutathione directly reduces an oxidoreductase in the endoplasmic reticulum of mammalian cells. *J. Biol. Chem.* **279**, 55341–55347
- Appenzeller-Herzog, C. (2011) Glutathione- and non-glutathione-based oxidant control in the endoplasmic reticulum. *J. Cell Sci.* **124**, 847–855
- Kim, S., Sideris, D. P., Sevier, C. S. and Kaiser, C. A. (2012) Balanced Ero1 activation and inactivation establishes ER redox homeostasis. *J. Cell Biol.* **196**, 713–725
- Sevier, C. S., Qu, H., Heldman, N., Gross, E., Fass, D. and Kaiser, C. A. (2007) Modulation of cellular disulfide-bond formation and the ER redox environment by feedback regulation of Ero1. *Cell* **129**, 333–344
- Heldman, N., Vonshak, O., Sevier, C. S., Vitu, E., Mehlman, T. and Fass, D. (2010) Steps in reductive activation of the disulfide-generating enzyme Ero1p. *Protein Sci.* **19**, 1863–1876
- Birk, J., Meyer, M., Aller, I., Hansen, H. G., Odermatt, A., Dick, T. P., Meyer, A. J. and Appenzeller-Herzog, C. (2013) Endoplasmic reticulum: reduced and oxidized glutathione revisited. *J. Cell Sci.*, doi: 10.1242/jcs.117218

- 33 Dias-Gunasekara, S., Gubbens, J., van Lith, M., Dunne, C., Williams, J. A., Katakly, R., Scoones, D., Laphorn, A., Bulleid, N. J. and Benham, A. M. (2005) Tissue-specific expression and dimerization of the endoplasmic reticulum oxidoreductase Ero1 $\beta$ . *J. Biol. Chem.* **280**, 33066–33075
- 34 Dias-Gunasekara, S., van Lith, M., Williams, J. A., Katakly, R. and Benham, A. M. (2006) Mutations in the FAD binding domain cause stress-induced misoxidation of the endoplasmic reticulum oxidoreductase Ero1 $\beta$ . *J. Biol. Chem.* **281**, 25018–25025
- 35 Appenzeller-Herzog, C. and Ellgaard, L. (2008) *In vivo* reduction-oxidation state of protein disulfide isomerase: the two active sites independently occur in the reduced and oxidized forms. *Antioxid. Redox Signal.* **10**, 55–64
- 36 Arnold, K., Bordoli, L., Kopp, J. and Schwede, T. (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**, 195–201
- 37 Masui, S., Vavassori, S., Fagioli, C., Sitia, R. and Inaba, K. (2011) Molecular bases of cyclic and specific disulfide interchange between human ERO1 $\alpha$  protein and protein-disulfide isomerase (PDI). *J. Biol. Chem.* **286**, 16261–16271
- 38 Ahmad, S., Gromiha, M., Fawareh, H. and Sarai, A. (2004) ASAView: database and tool for solvent accessibility representation in proteins. *BMC Bioinformatics* **5**, 51
- 39 Fraczekiewicz, R. and Braun, W. (1998) Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J. Comput. Chem.* **19**, 319–333
- 40 Hansen, R. E. and Winther, J. R. (2009) An introduction to methods for analyzing thiols and disulfides: Reactions, reagents, and practical considerations. *Anal. Biochem.* **394**, 147–158
- 41 Lind, C., Gerdes, R., Hamnell, Y., Schuppe-Koistinen, I., von Lowenhillem, H. B., Holmgren, A. and Cotgreave, I. A. (2002) Identification of S-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch. Biochem. Biophys.* **406**, 229–240
- 42 Otsu, M., Bertoli, G., Fagioli, C., Guerini-Rocco, E., Nerini-Molteni, S., Ruffato, E. and Sitia, R. (2006) Dynamic retention of Ero1 $\alpha$  and Ero1 $\beta$  in the endoplasmic reticulum by interactions with PDI and ERp44. *Antioxid. Redox Signal.* **8**, 274–282
- 43 Sato, Y., Kojima, R., Okumura, M., Hagiwara, M., Masui, S., Maegawa, K., Saiki, M., Horibe, T., Suzuki, M. and Inaba, K. (2013) Synergistic cooperation of PDI family members in peroxiredoxin 4-driven oxidative protein folding. *Sci. Rep.* **3**, 2456
- 44 Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A. and Mori, K. (2007) Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6 $\alpha$  and XBP1. *Dev. Cell* **13**, 365–376
- 45 Zito, E., Chin, K. T., Blais, J., Harding, H. P. and Ron, D. (2010) ERO1- $\beta$ , a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. *J. Cell Biol.* **188**, 821–832
- 46 Wright, J., Birk, J., Haataja, L., Liu, M., Ramming, T., Weiss, M. A., Appenzeller-Herzog, C. and Arvan, P. (2013) Endoplasmic reticulum oxidoreductin-1 $\alpha$  (ero1 $\alpha$ ) improves folding and secretion of mutant proinsulin and limits mutant proinsulin-induced ER stress. *J. Biol. Chem.* **288**, 31010–31018
- 47 Araki, K. and Nagata, K. (2011) Functional *in vitro* analysis of the ERO1 protein and protein-disulfide isomerase pathway. *J. Biol. Chem.* **286**, 32705–32712

---

Received 19 November 2013/8 January 2014; accepted 28 January 2014

---

Published as Immediate Publication 25 February 2014, doi 10.1042/BSR20130124

---



OPEN ACCESS

## SUPPLEMENTARY DATA

# Biochemical evidence that regulation of Ero1 $\beta$ activity in human cells does not involve the isoform-specific cysteine 262

Henning G. HANSEN\*<sup>1</sup>, Cecilie L. SØLTOFT\*, Jonas D. SCHMIDT\*<sup>2</sup>, Julia BIRK†, Christian APPENZELLER-HERZOG\*† and Lars ELLGAARD\*<sup>3</sup>

\*Department of Biology, University of Copenhagen, 2200 Copenhagen, Denmark

†Department of Pharmaceutical Sciences, University of Basel, 4056 Basel, Switzerland

Supplementary Figure S1 is on the following page.

<sup>1</sup> Present address: Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2970 Hørsholm, Denmark.

<sup>2</sup> Present address: Department of International Health, Immunology and Microbiology, University of Copenhagen, 2200 Copenhagen, Denmark.

<sup>3</sup> To whom correspondence should be addressed (email: lellgaard@bio.ku.dk).



		42 44	
Xt	1	-----MEVNRVVPVLVMMIISLSPSTEDT--ETTEREAKKESRAVSEQSCGCHITGVLD	DDCDVBSIDAFNNKIFP
Ac	1	-----ITGVLD	DDCDIISIDFNNKIFP
Md	1	MSRAGRRS--RAGAAAQVQLWVTLSCLTAA-----EAQITGVLD	DDCDIISIDSFNTYKIFP
Mm	1	MSPGFRAVGTGGAAAQVQLVTLSTLSSLV-----KTQITGVLD	DDCDIISIDFNTYKIFP
Cf	1	MRPGARPAGAGGAAAAQVLLTLSTLSSLV-----EAQITGVLD	DDCDIISIDFNTYKIFP
Hs	1	MSQGVRRAGAGQGVAAVQLVTLSTLSSLV-----EAQITGVLD	DDCDIISIDFNTYKIFP
Dr	1	MLHRTQTHGLVILMSLICGYFCSGWGFQNAKSKSTQKEPVYGHTEDELQSC	CHITGVLD
Ol	1	-----VRLAMSTANVC-VWVQRILT-----LSWKLP	CLLOITGVLD
Tr	1	-----RYCETFILSRGI-----DGSDIVSSAQITGE	LDDCDVBSIDFNNKIFP
		81 90 95 100 130	
Xt	73	KLKLOERDYFRYYKVNLRKPCFWAEDGHCSIKDCHVEPCPEKVPVGIKAGNYN	---YSLER-NSEKIDACEQANKL
Ac	26	KIOKLOERDYFRYYKVNLRKPCFWAEDGHCSIKDCHVEPCPEKVPVGIKAGNAN	---YSKIA-NHTKEIDACEQANKL
Md	58	KIOKLLERDYFRYYKVNLRKPCFWAEDGHCSIKDCHVEPCPEKVPVGIKAGNSN	---YSKAA-NHTKEIDACEQANKL
Mm	60	KIKKLOERDYFRYYKVNLRKPCFWAEDGHCSIKDCHVEPCPEKVPVGIKAGRSN	---YSQAA-NSTKEIDACEQANKL
Cf	60	KIKKLOERDYFRYYKVNLRKPCFWAEDGHCSIKDCHVEPCPEKVPVGIKAGSSN	---YSKVA-NNTKEIDACEQANKL
Hs	60	KIKKLOERDYFRYYKVNLRKPCFWAEDGHCSIKDCHVEPCPEKVPVGIKAGHSN	---YLKMA-NNTKEIDACEQANKL
Dr	81	HIRKLTERDYFRYYKVNLRKPCFWPDGSHCSIKDCHVEPCPEKVPVGIKSGNYN	---YSHAA-NTVSEIRECEQAHKL
Ol	55	QIKKLTERDYFRYYKVNLRKPCFWPDGSHCSIKDCHVEPCPEKVPVGIKSGNYN	KKHYSQSNNKISSEKFILLHKT
Tr	48	RIKKLTERDYFRYYKVNLRKPCFWPDGSHCSIKDCHVEPCPEKVPVGIKSGNYN	---YSQVA-NLMADTECEQANKL
		165 207	
Xt	150	GAIN--STLSNQSKEAFIDWARYDDAQDHFCELDDBRSPDQVYVDLLNPERYTG	YKGPSAWRVWNSIYEENCFKPRSVY
Ac	103	GAIN--STLSNQSKEAFIDWARYDDSDHFCELDDBRSPDAQYVDLLNPERYTG	YKGPSAWRVWNSIYEENCFKPRSVY
Md	135	GAIN--STLSNQSKEAFIDWARYDDSDHFCELDDBRSPAVQYVDLLNPERYTG	YKGPSAWRVWNSIYEENCFKPRSVY
Mm	137	GAIN--STLSNQSKEAFIDWARYDDSDHFCELDDBRSPAAQYVDLLNPERYTG	YKGPSAWRVWNSIYEENCFKPRSVY
Cf	137	GAIN--STLSNQSKEAFIDWARYDDSDHFCELDDBRSPAAQYVDLLNPERYTG	YKGPSAWRVWNSIYEENCFKPRSVY
Hs	137	GAIN--STLSNQSKEAFIDWARYDDSDHFCELDDBRSPAAQYVDLLNPERYTG	YKGPSAWRVWNSIYEENCFKPRSVY
Dr	158	GAIN--STLSNQSKEAFADWAHDDAQDHFCELDDBRSPSEYVDLLNPERYTG	YKGPSAWRVWNSIYEENCFKPRSVY
Ol	135	NSINYFSCYSNQSKEAFADWTRHDDAQDHFCELDDBRSPDAEYVDLLNPERYTG	YKGPSAWRVWNSIYEENCFKPRSVY
Tr	125	GAIN--STLSNQSKEAFADWARHDDAQDHFCELDDBRSPAAEYVDLLNPERYTG	YKGPSAWRVWNSIYEENCFKPRSVY
		240 262	
Xt	228	RPLNPLAPSRGDDGGSFYTWLEGLCLEKRVFYRLISGLHASINLHLCAKYLLE	ETWGRFRCINVEFTIRFDPSETKS
Ac	181	RPLNPLAPSKGDDGGSFYTWLEGLCLEKRVFYRLISGLHASINLHLCAKYLLE	ETWGRFRCINVEFTIRFDPSETKS
Md	213	RPLNPLAPSRGDDGGSFYTWLEGLCLEKRVFYRLISGLHASINLHLCAKYLLE	ETWGRFRCINVEFTIRFDPSETKS
Mm	215	RPLNPLAPSRGDDGGSFYTWLEGLCLEKRVFYRLISGLHASINLHLCAKYLLE	ETWGRFRCINVEFTIRFDPSETKS
Cf	215	RPLNPLAPSRGDDGGSFYTWLEGLCLEKRVFYRLISGLHASINLHLCAKYLLE	ETWGRFRCINVEFTIRFDPSETKS
Hs	215	RPLNPLAPSRGDDGGSFYTWLEGLCLEKRVFYRLISGLHASINLHLCAKYLLE	ETWGRFRCINVEFTIRFDPSETKS
Dr	236	RPLNPLAPVRGDDGGSFYTWLEGLCLEKRVFYRLISGLHSSINLHLCAKYLLE	DEGWGKEVWGPVNEHFRFDPSETKS
Ol	215	RPLNPLAPSRGDDGGSFYTWLEGLCLEKRVFYRLISGLHSSINLHLCAEYLL	DEGWGRAVWGPNIHFRFDPSETKS
Tr	203	RPLNPLAPSRGDDGGSFYTWLEGLCLEKRVFYRLISGLHSSINLHLCDHLL	DEGWGRSVWGEDLCEFRFDPSETKS
		390 393 396	
Xt	388	EEFRLHFNISRIMDCVGCCKRLWGKLQTOGLGTALKILFSEKEIQCLPEN	SPSKGFQLTRQEIIVALLNAFGR
Ac	341	EELRLHFNISRIMDCVGCCKRLWGKLQTOGLGTALKILFSEKEIQCLPEN	SPSKGFQLTRQEIIVALLNAFGR
Md	373	EEFRLHFNISRIMDCVGCCKRLWGKLQTOGLGTALKILFSEKEIQCLPEN	SPSKGFQLTRQEIIVALLNAFGR
Mm	375	EEFRLHFNISRIMDCVGCCKRLWGKLQTOGLGTALKILFSEKEIQCLPEN	SPSKGFQLTRQEIIVALLNAFGR
Cf	375	EEFRLHFNISRIMDCVGCCKRLWGKLQTOGLGTALKILFSEKEIQCLPEN	SPSKGFQLTRQEIIVALLNAFGR
Hs	375	EEFRLHFNISRIMDCVGCCKRLWGKLQTOGLGTALKILFSEKEIQCLPEN	SPSKGFQLTRQEIIVALLNAFGR
Dr	396	EEFRLHFNISRIMDCVGCCKRLWGKLQTOGLGTALKILFSEKEIQCLPEN	SPSKGFQLTRQEIIVALLNAFGR
Ol	375	EEFRLHFNISRIMDCVGCCKRLWGKLQTOGLGTALKILFSEKEIQCLPEN	SPSKGFQLTRQEIIVALLNAFGR
Tr	363	EEFRLHFNISRIMDCVGCCKRLWGKLQTOGLGTALKILFSEKEIQCLPEN	SPSKGFQLTRQEIIVALLNAFGR
		468 476 483 490 497 504 511 518 525 532 539 546 553 560 567 574 581 588 595 602 609 616 623 630 637 644 651 658 665 672 679 686 693 700 707 714 721 728 735 742 749 756 763 770 777 784 791 798 805 812 819 826 833 840 847 854 861 868 875 882 889 896 903 910 917 924 931 938 945 952 959 966 973 980 987 994 1001 1008 1015 1022 1029 1036 1043 1050 1057 1064 1071 1078 1085 1092 1099 1106 1113 1120 1127 1134 1141 1148 1155 1162 1169 1176 1183 1190 1197 1204 1211 1218 1225 1232 1239 1246 1253 1260 1267 1274 1281 1288 1295 1302 1309 1316 1323 1330 1337 1344 1351 1358 1365 1372 1379 1386 1393 1400 1407 1414 1421 1428 1435 1442 1449 1456 1463 1470 1477 1484 1491 1498 1505 1512 1519 1526 1533 1540 1547 1554 1561 1568 1575 1582 1589 1596 1603 1610 1617 1624 1631 1638 1645 1652 1659 1666 1673 1680 1687 1694 1701 1708 1715 1722 1729 1736 1743 1750 1757 1764 1771 1778 1785 1792 1799 1806 1813 1820 1827 1834 1841 1848 1855 1862 1869 1876 1883 1890 1897 1904 1911 1918 1925 1932 1939 1946 1953 1960 1967 1974 1981 1988 1995 2002 2009 2016 2023 2030 2037 2044 2051 2058 2065 2072 2079 2086 2093 2100 2107 2114 2121 2128 2135 2142 2149 2156 2163 2170 2177 2184 2191 2198 2205 2212 2219 2226 2233 2240 2247 2254 2261 2268 2275 2282 2289 2296 2303 2310 2317 2324 2331 2338 2345 2352 2359 2366 2373 2380 2387 2394 2401 2408 2415 2422 2429 2436 2443 2450 2457 2464 2471 2478 2485 2492 2499 2506 2513 2520 2527 2534 2541 2548 2555 2562 2569 2576 2583 2590 2597 2604 2611 2618 2625 2632 2639 2646 2653 2660 2667 2674 2681 2688 2695 2702 2709 2716 2723 2730 2737 2744 2751 2758 2765 2772 2779 2786 2793 2800 2807 2814 2821 2828 2835 2842 2849 2856 2863 2870 2877 2884 2891 2898 2905 2912 2919 2926 2933 2940 2947 2954 2961 2968 2975 2982 2989 2996 3003 3010 3017 3024 3031 3038 3045 3052 3059 3066 3073 3080 3087 3094 3101 3108 3115 3122 3129 3136 3143 3150 3157 3164 3171 3178 3185 3192 3199 3206 3213 3220 3227 3234 3241 3248 3255 3262 3269 3276 3283 3290 3297 3304 3311 3318 3325 3332 3339 3346 3353 3360 3367 3374 3381 3388 3395 3402 3409 3416 3423 3430 3437 3444 3451 3458 3465 3472 3479 3486 3493 3500 3507 3514 3521 3528 3535 3542 3549 3556 3563 3570 3577 3584 3591 3598 3605 3612 3619 3626 3633 3640 3647 3654 3661 3668 3675 3682 3689 3696 3703 3710 3717 3724 3731 3738 3745 3752 3759 3766 3773 3780 3787 3794 3801 3808 3815 3822 3829 3836 3843 3850 3857 3864 3871 3878 3885 3892 3899 3906 3913 3920 3927 3934 3941 3948 3955 3962 3969 3976 3983 3990 3997 4004 4011 4018 4025 4032 4039 4046 4053 4060 4067 4074 4081 4088 4095 4102 4109 4116 4123 4130 4137 4144 4151 4158 4165 4172 4179 4186 4193 4200 4207 4214 4221 4228 4235 4242 4249 4256 4263 4270 4277 4284 4291 4298 4305 4312 4319 4326 4333 4340 4347 4354 4361 4368 4375 4382 4389 4396 4403 4410 4417 4424 4431 4438 4445 4452 4459 4466 4473 4480 4487 4494 4501 4508 4515 4522 4529 4536 4543 4550 4557 4564 4571 4578 4585 4592 4599 4606 4613 4620 4627 4634 4641 4648 4655 4662 4669 4676 4683 4690 4697 4704 4711 4718 4725 4732 4739 4746 4753 4760 4767 4774 4781 4788 4795 4802 4809 4816 4823 4830 4837 4844 4851 4858 4865 4872 4879 4886 4893 4900 4907 4914 4921 4928 4935 4942 4949 4956 4963 4970 4977 4984 4991 4998 5005 5012 5019 5026 5033 5040 5047 5054 5061 5068 5075 5082 5089 5096 5103 5110 5117 5124 5131 5138 5145 5152 5159 5166 5173 5180 5187 5194 5201 5208 5215 5222 5229 5236 5243 5250 5257 5264 5271 5278 5285 5292 5299 5306 5313 5320 5327 5334 5341 5348 5355 5362 5369 5376 5383 5390 5397 5404 5411 5418 5425 5432 5439 5446 5453 5460 5467 5474 5481 5488 5495 5502 5509 5516 5523 5530 5537 5544 5551 5558 5565 5572 5579 5586 5593 5600 5607 5614 5621 5628 5635 5642 5649 5656 5663 5670 5677 5684 5691 5698 5705 5712 5719 5726 5733 5740 5747 5754 5761 5768 5775 5782 5789 5796 5803 5810 5817 5824 5831 5838 5845 5852 5859 5866 5873 5880 5887 5894 5901 5908 5915 5922 5929 5936 5943 5950 5957 5964 5971 5978 5985 5992 5999 6006 6013 6020 6027 6034 6041 6048 6055 6062 6069 6076 6083 6090 6097 6104 6111 6118 6125 6132 6139 6146 6153 6160 6167 6174 6181 6188 6195 6202 6209 6216 6223 6230 6237 6244 6251 6258 6265 6272 6279 6286 6293 6300 6307 6314 6321 6328 6335 6342 6349 6356 6363 6370 6377 6384 6391 6398 6405 6412 6419 6426 6433 6440 6447 6454 6461 6468 6475 6482 6489 6496 6503 6510 6517 6524 6531 6538 6545 6552 6559 6566 6573 6580 6587 6594 6601 6608 6615 6622 6629 6636 6643 6650 6657 6664 6671 6678 6685 6692 6699 6706 6713 6720 6727 6734 6741 6748 6755 6762 6769 6776 6783 6790 6797 6804 6811 6818 6825 6832 6839 6846 6853 6860 6867 6874 6881 6888 6895 6902 6909 6916 6923 6930 6937 6944 6951 6958 6965 6972 6979 6986 6993 7000 7007 7014 7021 7028 7035 7042 7049 7056 7063 7070 7077 7084 7091 7098 7105 7112 7119 7126 7133 7140 7147 7154 7161 7168 7175 7182 7189 7196 7203 7210 7217 7224 7231 7238 7245 7252 7259 7266 7273 7280 7287 7294 7301 7308 7315 7322 7329 7336 7343 7350 7357 7364 7371 7378 7385 7392 7399 7406 7413 7420 7427 7434 7441 7448 7455 7462 7469 7476 7483 7490 7497 7504 7511 7518 7525 7532 7539 7546 7553 7560 7567 7574 7581 7588 7595 7602 7609 7616 7623 7630 7637 7644 7651 7658 7665 7672 7679 7686 7693 7700 7707 7714 7721 7728 7735 7742 7749 7756 7763 7770 7777 7784 7791 7798 7805 7812 7819 7826 7833 7840 7847 7854 7861 7868 7875 7882 7889 7896 7903 7910 7917 7924 7931 7938 7945 7952 7959 7966 7973 7980 7987 7994 8001 8008 8015 8022 8029 8036 8043 8050 8057 8064 8071 8078 8085 8092 8099 8106 8113 8120 8127 8134 8141 8148 8155 8162 8169 8176 8183 8190 8197 8204 8211 8218 8225 8232 8239 8246 8253 8260 8267 8274 8281 8288 8295 8302 8309 8316 8323 8330 8337 8344 8351 8358 8365 8372 8379 8386 8393 8400 8407 8414 8421 8428 8435 8442 8449 8456 8463 8470 8477 8484 8491 8498 8505 8512 8519 8526 8533 8540 8547 8554 8561 8568 8575 8582 8589 8596 8603 8610 8617 8624 8631 8638 8645 8652 8659 8666 8673 8680 8687 8694 8701 8708 8715 8722 8729 8736 8743 8750 8757 8764 8771 8778 8785 8792 8799 8806 8813 8820 8827 8834 8841 8848 8855 8862 8869 8876 8883 8890 8897 8904 8911 8918 8925 8932 8939 8946 8953 8960 8967 8974 8981 8988 8995 9002 9009 9016 9023 9030 9037 9044 9051 9058 9065 9072 9079 9086 9093 9100 9107 9114 9121 9128 9135 9142 9149 9156 9163 9170 9177 9184 9191 9198 9205 9212 9219 9226 9233 9240 9247 9254 9261 9268 9275 9282 9289 9296 9303 9310 9317 9324 9331 9338 9345 9352 9359 9366 9373 9380 9387 9394 9401 9408 9415 9422 9429 9436 9443 9450 9457 9464 9471 9478 9485 9492 9499 9506 9513 9520 9527 9534 9541 9548 9555 9562 9569 9576 9583 9590 9597 9604 9611 9618 9625 9632 9639 9646 9653 9660 9667 9674 9681 9688 9695 9702 9709 9716 9723 9730 9737 9744 9751 9758 9765 9772 9779 9786 9793 9800 9807 9814 9821 9828 9835 9842 9849 9856 9863 9870 9877 9884 9891 9898 9905 9912 9919 9926 9933 9940 9947 9954 9961 9968 9975 9982 9989 9996 10003 10010 10017 10024 10031 10038 10045 10052 10059 10066 10073 10080 10087 10094 10101 10108 10115 10122 10129 10136 10143 10150 10157 10164 10171 10178 10185 10192 10199 10206 10213 10220 10227 10234 10241 10248 10255 10262 10269 10276 10283 10290 10297 10304 10311 10318 10325 10332 10339 10346 10353 10360 10367 10374 10381 10388 10395 10402 10409 10416 10423 10430 10437 10444 10451 10458 10465 10472 10479 10486 10493 10500 10507 10514 10521 10528 10535 10542 10549 10556 10563 10570 10577 10584 10591 10598 10605 10612 10619 10626 10633 10640 10647 10654 10661 10668 10675 10682 10689 10696 10703 10710 10717 10724 10731 10738 10745 10752 10759 10766 10773 10780 10787 10794 10801 10808 10815 10822 10829 10836 10843 10850 10857 10864 10871 10878 10885 10892 10899 10906 10913 10920 10927 10934 10941 10948 10955 10962 10969 10976 10983 10990 10997 11004 11011 11018 11025 11032 11039 11046 11053 11060 11067 11074 11081 11088 11095 11102 11109 11116 11123 11130 11137 11144 11151 11158 11165 11172 11179 11186 11193 11200 11207 11214 11221 11228 11235 11242 11249 11256 11263 11270 11277 11284 11291 11298 11305 11312 11319 11326 11333 11340 11347 11354 11361 11368 11375 11382 11389 11396 11403 11410 11417 11424 11431 11438 11445 11452 11459 11466 11473 11480 11487 11494 11501 11508 11515 11522 11529 11536 11543 11550 11557 11564 11571 11578 11585 11592 11599 11606 11613 11620 11627 11634 11641 11648 11655 11662 11669 11676 11683 11690 11697 11704 11711 11718 11725 11732 11739 11746 11753 11760 11767 11774 11781 11788 11795 11802 11809 11816 11823 11830 11837 11844 11851 11858 11865 11872 11879 11886 11893 11900 11907 11914 11921 11928 11935 11942 11949 11956 11963 11970 11977 11984 11991 11998 12005 12012 12019 12026 12033 12040 12047 12054 12061 12068 12075 12082 12089 12096 12103 12110 12117 12124 12131 12138 12145 12152 12159 12166 12173 12180 12187 12194 12201 12208 12215 12222 12229 12236 12243 12250 12257 12264 12271 12278 12285 12292 12299 12306 12313 12320 12327 12334 12341 12348 12355 12362 12369 12376 12383 12390 12397 12404 12411 12418 12425 12432 12439 12446 12453 12460 12467 12474 12481 12488 12495 12502 12509 12516 12523 12530 12537 12544 12551 12558 12565 12572 12579 12586 12593 126	



**Figure S1 Evolutionary conservation of Ero1 $\beta$** 

A multiple sequence alignment of Ero1 $\beta$  orthologs was performed with Muscle [1] using the following UniProt entries. *Xenopus tropicalis* (frog; F6ULN4); *Gallus gallus* (chicken; E1C917); *Anolis carolinensis* (lizard; G1KAL4); *Monodelphis domestica* (opossum; F7CL82); *Mus musculus* (mouse; Q8R2E9); *Canis familiaris* (dog; F1Q091); *Homo sapiens* (human; Q86YB8); *Danio rerio* (zebrafish; E7F2A8); *Oryzias latipes* (rice fish; H2L719); *Takifugu rubripes* (pufferfish; H2TT03). Black boxes indicate amino acid identities and grey boxes show amino acid similarities when found in at least seven of the nine sequences. The human sequence is shown in boldface, cysteine residues are shown in red colour and amino acid position of the cysteine residues in the human sequence is indicated above the alignment.

---

**REFERENCE**

- 1 Edgar, R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797

---

Received 19 November 2013/8 January 2014; accepted 28 January 2014

---

Published as Immediate Publication 25 February 2014, doi 10.1042/BSR20130124

---